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Article *in* Applied Microbiology and Biotechnology · September 2012

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Appl Microbiol Biotechnol (2013) 97:3007–3016
DOI 10.1007/s00253-012-4403-7

Improving the safety of viral DNA vaccines: development of vectors containing both 5′ and 3′ homologous regulatory sequences from non-viral origin

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Received: 1 June 2012 / Revised: 29 August 2012 / Accepted: 4 September 2012 / Published online: 23 September 2012

Abstract Although some DNA vaccines have proved to be very efficient in field trials, their authorisation still remains limited to a few countries. This is in part due to safety issues because most of them contain viral regulatory sequences to driving the expression of the encoded antigen. This is the case of the only DNA vaccine against a fish rhabdovirus (a negative ssRNA virus), authorised in Canada, despite the important economic losses that these viruses cause to aquaculture all over the world. In an attempt to solve this problem and using as a model a non-authorised, but efficient DNA vaccine against the fish rhabdovirus, viral haemorrhagic septicaemia virus (VHSV), we developed a plasmid construction containing regulatory sequences exclusively from fish origin. The result was an "all-fish vector", named pJAC-G, containing 5′ and 3′ regulatory sequences of β-acting genes from carp and zebrafish, respectively. In vitro and in vivo, pJAC-G drove a successful expression of the VHSV glycoprotein G (G), the only antigen of the virus conferring in vivo protection. Furthermore, and by means of in vitro fusion assays, it was confirmed that G protein expressed from pJAC-G was fully functional. Altogether, these results suggest that DNA vaccines containing host-homologous gene regulatory sequences might be useful for developing safer DNA vaccines, while they also might be useful for basic studies.

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Keywords Terminators · Vector regulatory sequences · Fish · DNA · Vaccines · Plasmid · VHSV · Rhabdovirus

Introduction

Vaccines for the prevention of human and animal infectious diseases are a major public health priority. Immunisation with DNA vaccines encoding key proteins involved in the immune response to pathogens has emerged as a major focus of vaccine research (Schleiss 2009) after three DNA vaccines were recently licensed for animal health applications (Salonius et al. 2007). These included two infectious disease vaccines, for West Nile virus in horses (Ft Dodge Animal Health), and infectious haematopoietic necrosis rhabdovirus (IHNV) in salmon (Novartis) and a melanoma cancer vaccine for dogs (Merial) (Williams et al. 2009).

In addition to the election of highly immunogenic target antigens, the vector design criteria are also crucial for the development of DNA vaccines. To date, most of DNA vaccine vectors, including those used in the authorised and commercialised DNA vaccines against west Nile virus (Laddy and Weiner 2006; Martin et al. 2007) and IHNV (Alonso et al. 2011; Salonius et al. 2007), rely on the 5′ regulatory sequences (enhancer/promoter sequences) of the human immediate early cytomegalovirus (CMV) gene (Belakova et al. 2007; Donnelly et al. 2005; Laddy and Weiner 2006; Liu et al. 1995). In addition, 3′ regulatory sequences (transcription terminators/polyadenylation signals) derived from other viruses, like those from the simian virus (SV40), are commonly present in DNA vaccine vectors (Williams et al. 2009). Despite the effectiveness of these systems, viral regulatory sequences constitute and impediment for their licensing and commercialisation in many

countries. For example, the use of the DNA vaccine based on the IHN rhabdovirus glycoprotein G gene licensed in Canada has been not authorised by the European Union, although the economic costs of rhabdoviruses caused diseases to the European salmonid aquaculture industry are estimated at about 40–50 million euros per year (Chico et al. 2009), and there are neither specific agents nor other efficient vaccines for the treatment or prevention of these diseases. Therefore, it is clear that an improved DNA vector design will facilitate DNA vaccines practical applications by increasing their safety (Williams et al. 2009).

As a first approach, vector constructs harbouring regulatory sequences derived from genes homologous to the DNA vaccine target animal species were considered. To evaluate this possibility and using as model a DNA vaccine against the viral haemorrhagic septicaemia virus (VHSV), we designed and developed a new DNA vaccine vector containing regulatory sequences only from fish origin ('all-fish vector'). For that, we first tested the efficacy of seven transcription terminator sequences selected from fish genes for the best in vitro expression of the glycoprotein G of VHSV. Next, the best expressing transcription terminator sequence was combined with a vector encoding the DNA sequence of VHSV glycoprotein G under the control of the 5′ regulatory sequences of carp β-actin gene, thus generating, the 'all-fish vector', pJAC-G. In vitro assays demonstrated that the glycoprotein G successfully expressed under the control of both 3′ and 5′ fish regulatory sequences. Furthermore, the expressed viral protein was fully functional as shown in fusion assays. Finally, this vector also mediated an efficacious in vivo expression of the antigen both at the transcription and protein levels. Taken together, these results suggest that DNA vaccines containing host-homologue gene regulatory sequences might be useful for developing safer DNA vaccines, while they also might be useful for basic studies.

Materials and methods

Expression vectors

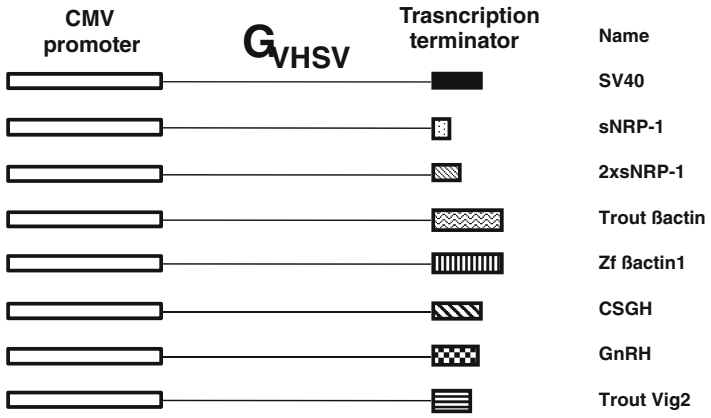
All of the vectors used in this work coded for the whole sequence of protein G of viral haemorrhagic septicaemia (VHSV) (Nuñez et al. 1998; Thiry et al. 1990) strain VHSV-0771, isolated in France from rainbow trout (*Oncorhynchus mykiss*) (LeBerre et al. 1977). The previously described pMCV1.4-G vector (Rocha et al. 2005) was used to construct the different fish terminator-containing plasmid constructs by replacing its simian virus 40 (SV40) transcription terminator sequence with synthetic sequences derived from terminators of fish origin. The terminator sequences were obtained from a recently described short terminator of soluble neuropilin-1 (sNRP-1) (McFarland et al. 2006), trout β-actin (accession no. AJ438158) (Johnson et al. 2004), zebrafish β-actin (accession no. BX005070), chinook salmon growth hormone CSGH (accession no. pFV3CAT) (Caldovic and Hackett 1995), tilapia gonadotropin releasing hormone GnRH (accession no. AF467291) (Farahmand et al. 2003) and trout Vig2 (accession no. AF290477) (Boudinot et al. 2001) (Fig. 1). The selected terminator, zebrafish β-actin (zf β-actin), was then cloned into the pAE6-G vector (Chico et al. 2009; Ortega-Villaizan et al. 2011) replacing the SV40 terminator. The regulatory sequences (2,577 base pairs, bp) of pAE6 vector (Brocal et al. 2006; Cheng et al. 2002) included the carp β-actin enhancer/promoter sequence as well as both the first exon (a non-coding exon) and first intron sequence from the carp β-actin gene (Liu et al. 1990) (GenBank accession no. M24113).

Construction of plasmids with fish terminators

All the selected terminators from GenBank fish sequences were chemically synthesized (BioS&T; Montreal, Canada) flanked with NheI and PciI and inserted into the pMCV1.4-G vector (Fig. 1) obtaining thus the different fish transcript terminator-containing vectors. *Escherichia coli* DH5alpha (Invitrogen) were then transformed by electroporation with each of the plasmid constructs. Large amounts of vector were prepared from *E. coli* pellets by using a modification of the Wizard plus Megaprep DNA purification system (Promega). After extraction, the plasmid were resolved on a 1 % agarose gel; then, the DNA bands corresponding with the theoretical size of supercoiled plasmid were excised and purified from the gel following standard procedures. The concentration of the DNA was then estimated by nanodrop ND1000 spectrophotometry measurements (Nanodrop Technologies Inc.). Vector solutions were adjusted to 0.5–1 mg/ml in water and kept frozen until used.

Cell cultures

Epithelioma papulosum cyprini (EPC) cells were obtained from the American Type Culture Collection (ATCC) collection (catalogue no. CRL-2872). They were recently found to be from fathead minnow (*Pimephales promelas*) (Winton et al. 2010). EPC cells were grown at 28 °C in RPMI Dutch modified cell culture medium 20 mM HEPES (Flow), 10 % foetal calf serum (FCS), 1 mM pyruvate, 2 mM glutamine, 50 μg/ml of gentamicin and 2.5 μg/ml of Fungizone. Likewise, RTG-2 cells (fibroblastic cell line derived from rainbow trout gonad), also purchased from ATCC (CCL-55), were maintained at 20 °C in a 5 % CO_2 atmosphere with minimal essential medium (MEM) (with Earle's salts) cell culture medium (Gibco) containing 10 % FCS (Sigma), 2 mM glutamine (Gibco) and 50 μg/ml neomycin sulphate (Sigma).



Name	Definition	bp	Acc. numbers	Position numbers
SV40	Simian Virus 40	202	pMCV1.4	2625-2826
sNRP-1	*soluble neuropilin-1	17	----------	----------
2xsNRP-1	*duplicated sNRP-1	34	----------	----------
Trout ßactin	Trout ßactin 3'UTR	688	AJ438158	1190-1879
Zf ßactin1	Zebrafish ßactin1 3'UTR	667	BX005070	14787-15453
CSGH	Chinook Salmon Growth Hormone	357	pFV3CAT	3547-3903
GnRH	Tilapia Gonadotropin Releasing Hormone	226	AF467291	5596-5820
Trout Vig2	Trout VHSV-Induced gene 2	134	AF290477	1561-1712

Fig. 1 Substitutions of SV40 transcription terminator with alternative fish terminators. The transcription terminator of the pMCV1.4-G vector was obtained from the SV40. It was substituted by the fish transcription terminators listed above by the corresponding chemical synthesis and subsequent subcloning into the pMCV1.4-G backbone. All the terminators have the consensus AATAA sequence. *AAATAAAATACGAAATG terminator sequence

Transfection assays

Cell transfection assays were performed as previously described (Brocal et al. 2006; Lopez et al. 2001). Briefly, EPC and RTG-2 cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsin (Sigma), washed, resuspended in culture medium supplemented with 10 % of FCS and dispensed into 96-well plates in approximately 5×10^4 EPC or 1.8×10^4 RTG-2 cells per well in a final volume of 100 μl. Transfections were carried out with the cells in suspension, right in the moment of the dispensing. The plasmids were added at different concentrations [250, 125 and 62 ng/well for the first screening, and 150 and 75 ng/well for the selected combination (the plasmid with the 5′ regulatory sequences from carp β-actin gene, pAE6, and containing the zf β-actin terminator)]. They were complexed with 0.3 μl of FuGene 6 (Roche, Barcelona, Spain) for EPC cells or FuGene HD (Roche, Barcelona, Spain) for RTG-2 cells, incubated for 30 min in 25 μl of RPMI-1640 containing 2 mM $CaCl_2$ and then added to each well in 100 μl of complete cell culture medium. Plates were further incubated at 20 °C for 2 days for EPC cells, or at 14 °C for 3 days for RTG-2.

Gene expression assays

Cells were transfected as described above. Plates were then incubated at 20 °C for 2 days for EPC or at 14 °C for 3 days for RTG-2, and the expression of VHSV G glycoprotein transcripts was then assessed by RT-qPCR. An RNeasy Plus minikit (Qiagen) was used for total RNA extraction following the manufacturer's instructions, and isolated RNAs were stored at −80 °C until used. One microgram of RNA, as estimated by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.), was used to obtain the cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed in an ABI PRISM 7300 System (Applied Biosystems, NJ). The internal reference to normalise data was the 18S rRNA (Applied Biosystems) for EPC and the cellular elongation factor 1 alpha (EF1-α) gene for RTG-2. Reactions were performed with 2 μl of cDNA reaction mixture, 900 nM each primer, 200 nM probe and 10 μl of TaqMan universal PCR master mix (Applied Biosystems) in 20 μl volume. The cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for

15 s and 60 °C for 1 min. Gene expression results were analysed by means of the $2^{-\Delta Ct}$ method (Martinez-Alonso et al. 2011) where ΔCt was determined by subtracting the 18S- or the EF1α-Ct value from the target Ct. Primers used were for EF-α: forward 5′-ACCCTCCTCTTGGTC GTTTC-3′, reverse 5′-TGATGACACCAACAGCAACA-3′ and probe 5′-GCTGTGCGTGACATGAGGCA-3′. Primers used for the VHSV G gene were forward 5′-GGGCCTT CCTTCTACTGGTACTC-3′, reverse 5′-CGGAATCCC GTAATTTGGAAT-3′ and probe 5′-CTGTTGCTGCAAG GCGTCCCCT-3′. For each condition, the Student *t* one-tail statistic associated *P* value was computed in relation to the pMCV1.4-G-pSV40 data. When indicated, results were expressed as percentage of transcript expression levels in the cell transfected with the plasmid pMCV1.4-G and calculated by the formula, (transcript level of VHSV G in cells transfected with a fish transcript terminator-containing vector/transcript level of VHSV G in cells transfected with pMCV1.4-G)×100.

Flow cytometry assays

Transfected cell monolayers were incubated with a cocktail of anti-gpG monoclonal antibodies (MAbs) (Mas et al. 2004) diluted 100-fold with RPMI and 10 % FCS for 1 h at 20 °C with occasional gentle agitation. After careful washing with RPMI 10 % FCS medium, the monolayers were incubated with rabbit anti-mouse Fab′2 IgG–FITC conjugate (Nordic) diluted 200-fold with RPMI and 10 % FCS for 30 min at 20 °C. After washing with RPMI 10 % FCS medium, the cell monolayers were detached and transferred to tubes by using FACS buffer consisting of PBS (100 mM Na_2HPO_4, 27 mM KCl, 17 mM KH_2PO_4, 1.3 M NaCl, pH 7.4), 0.1 % bovine serum albumin, 0.01 % NNa_3 and 50 mM EDTA. Transfections with pMCV1.4-gpG at the established concentrations were included in each experiment to correct for variations between experiments, representing each value the 100 % of transfection for each dilution. The number of cells expressing the G protein in the membrane of transfected cells was estimated in 10,000 cells by using a FACScanto II apparatus (Becton Dickinson) and the software FACSDiva v6.3.1 (Becton Dickinson). Fluorescence histograms versus number of cells showed two peaks of fluorescence separated by a threshold value of significant FL1 fluorescence above the background fluorescence obtained with pMCV1.4 transfected cells between 20 and 30 arbitrary fluorescence units depending on the experiment. The percentage of the fluorescent cells were calculated by the formula, number of fluorescent cells above the threshold/total number of cells×100 (Ruiz et al. 2008). The results are expressed as means and standard deviations from two different independent experiments.

Immunofluorescence assays

To detect the presence of VHSV gpG on the cell membranes, RTG-2 and EPC cells were transfected, grown in 96-well plates, fixed with 4 % paraformaldehyde in PBS (15 min at room temperature) and incubated with a cocktail of anti-gpG MAbs (Mas et al. 2004), diluted 200-fold for 2 h at room temperature. After a wash with PBS, 300 μl of fluorescein-labelled rabbit anti-mouse IgG Ab (Sigma) diluted 200-fold was added per well and incubated for 45 min. Stained cells were viewed and photographed with an inverted fluorescence microscope (Nikon Eclipse TE2000-U) equipped with a digital camera (Nikon DS-1QM).

Fusion assays

RTG-2 and EPC cells were transfected and grown on 96-well plates as indicated above. After 48 h in the case of EPC or 72 h for RTG-2, the cell culture medium was removed, cells were washed and the fusion was triggered by incubating the cells with fusion medium (Mas et al. 2002) at pH6 for 30 min at 14 °C. Monolayers were then washed and incubated with fusion medium at pH 7.5 for 2 h at room temperature. To evaluate the fusion, cells were fixed with cold methanol, dried and stained with Giemsa (5 mg/ml in PBS) (Estepa et al. 2001). Cells were viewed and photographed with an inverted fluorescence microscope (Nikon) provided with a digital camera (Nikon DS-1QM). As fusion positive control, EPC and RTG-2 cells infected with VHSV were used.

Fish maintenance and DNA immunisation protocol

Rainbow trout (*Oncorhynchus mykiss*) of approximately 5–6 cm in length obtained from a VHSV-free commercial farm (Lillogen, Leon, Spain) were maintained in 50-l tanks at the University Miguel Hernandez facilities at 12–14 °C with a re-circulating dechlorinated-water system and fed daily with a commercial diet (Trow, Leon, Spain). Prior to experiments, fish were acclimatised to laboratory conditions for 2 weeks. For DNA immunisation, trout were anaesthetised by immersion in 50 μg/ml buffered tricaine methanesulphonate (MS-222; Sigma) prior to handling and then divided into groups. Groups were intramuscularly injected with one of the following: 50 μl of PBS (non-immunised or control fish) or 50 μl of PBS containing 2 μg of pAE6-G or pJAC-G plasmids. At days3 and 12 post-immunisation (p.i.), three fish from each group were sacrificed by overexposure to MS-222 and tissue from muscle (injection site) removed.

In vivo detection of VHSV G expression

Three and 12 days after the DNA immunisation, the levels of expression of VHSV G protein in the trout skeletal

muscle were analysed at both transcriptional and protein levels by quantitative real time RT-PCR (qRT-PCR) and ELISA, respectively. The samples of muscle tissue were taken from the site of injection and processed for qRT-PCR as indicated for transfected cells or homogenate for ELISA assays. For determining the G_{VHSV} expression in injected trout muscle by ELISA, about 20 mg of muscle tissue was homogenised in 300 μl of distilled water and clarified by centrifugation as previously described (Hwang et al. 2003). Protein was adjusted to 0.1 mg/ml as estimated by a NanoDrop spectrophotometer 2000c (Thermo Fisher Scientific Inc.) and frozen at −20 °C until use. One hundred microliters of muscle homogenates per well (~10 μg of protein) was dried (in 96-well polystyrene plates, Dynatech) by incubation overnight at 37 °C. Before use, the coated plates were incubated for 1 h at room temperature with 3 % dry milk in dilution buffer, washed and then incubated for 120 min at room temperature with 100 μl/well of the MAb anti-G_{VHSV} I16, a non-conformation-dependent MAb, diluted 300-fold in dilution buffer. After washing with distilled water, 100 μl/well of a peroxidase-labelled goat anti-mouse IgG Ab (Sigma) was added and the 1-Step Ultra TMB-ELISA (Thermo Scientific) was used to develop the peroxidase reaction. Sulphuric acid was finally added to stop the reaction and the absorbance was measured at 450 nm.

Statistical analysis

Means of replicate experiments were compared by using an unpaired, two-tailed Student's *t* test assuming unequal variance. *P* values <0.05 were considered to be statistically significant.

Results

Expression analysis by RT-qPCR for the screening of transcription terminator sequences from fish genes

For the screening of expression of fish transcription terminator sequences (Fig. 1), we used the pMCV 1.4-G as vector backbone (Rocha et al. 2005), encoding the DNA sequence of the VHSV glycoprotein G under the control of the CMV promoter and the SV40 transcription terminator. The seven terminator-containing vectors were then obtained by replacing the SV40 transcription terminator from pMCV1.4-G with synthetic sequences derived from terminators of fish origin. The screening of expression was carried out by RT-qPCR in two fish cell lines from different origin, one from cold water salmonids (RTG-2) and another from warm water cyprinids (EPC). No significant differences in G transcript levels were observed among the different fish terminator-containing vectors in either of the two cell lines (Fig. 2a). However, differences were found using different DNA concentrations (250, 125 and 62 ng/well) to transfect the RTG-2 cells. Yet, in the RTG-2 the highest concentration used (250 ng/well) yielded lower levels of G transcripts. No differences in cell viability were observed when transfection was carried out at the different DNA concentrations or among the plasmid used. Moreover, the highest levels of glycoprotein transcript expression in RTG-2 cells were found for the zf β-actin, GNRH and Vig2 terminators while in the EPC cell line, most of the levels of expression were 10-fold higher than in the RTG-2 cell line (Fig. 2b).

Since (1) none of the terminator-containing vectors (Fig. 1) showed comparable results in both cell lines (Fig. 2), and (2) the selected fish transcription terminator sequence should be included in a vector containing 5′ regulatory sequences from carp β-actin gene (pAE6), we chose the zf β-actin terminator to continue with the study in order to develop an expression system as homologous as possible.

In vitro VHSV G expression in pJAC-G transfected cells

To construct the all-fish vector encoding the sequence of VHSV G glycoprotein, the SV40 transcription terminator sequence was excised from the pAE6-G vector and replaced with the terminator from the zf β-actin gene. The resulting construct, pJAC-G, was then used to transfect RTG-2 and EPC cells and the expression levels of the G gene evaluated by RT-qPCR and flow cytometry. To compare the expression levels, RTG-2 and EPC were transfected with pAE6-G, a vector that contains the SV40 transcription terminator sequence. The results of these transfection assays showed that the transcript expression levels of the G gene in the RTG-2 cell line were 2- to 4-fold higher in the cells transfected with the pAE6-G than with the pJAC-G vector (Fig. 3a). In contrast, the percentage of G glycoprotein expressing cells in transfected RTG-2 cell cultures was similar regardless of the DNA concentration or VHSV-G containing plasmid used (Fig. 3b), suggesting a lack of direct correlation between transcript and protein expression levels.

EPC cells, on the other hand, showed a similar expression of the G glycoprotein at both transcript and proteins levels regardless of the VHSV G plasmid or concentration used (Fig. 3d, e). In this case, the maximum percentage of G glycoprotein expressing cells reached over 22 % and the intensity of fluorescence was higher than in transfected RTG-2 cells (Fig. 3c, f).

Cellular localisation of the G glycoprotein in pJAC-G transfected cells

It is known that the full-length G glycoprotein of VHSV remains associated to the plasmatic cell membrane (Walker

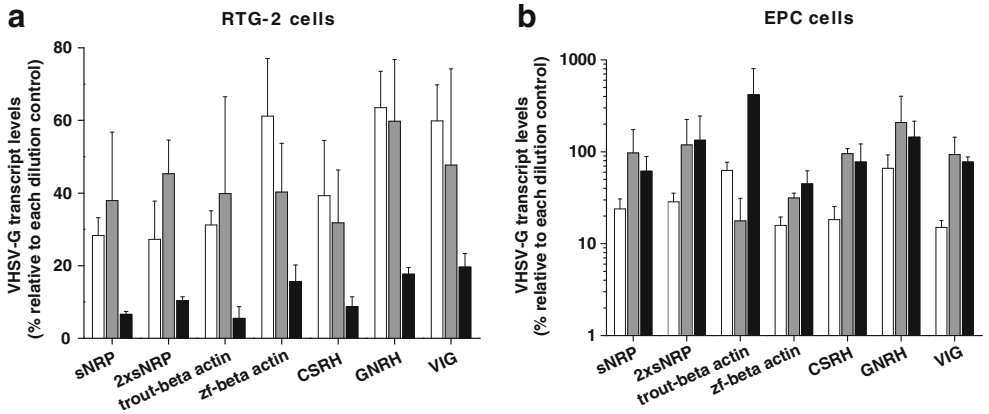


Fig. 2 Transcript levels of the G gene in EPC and RTG-2 cells transfected with pMCV1.4-G with different terminators from fish origin. The different constructs coding for the G protein of VHSV under the control of the CMV promoter and the different fish terminators (Fig. 1) were transfected into EPC (**a**) or RTG-2 (**b**) cell lines. Each construct was assayed at 62 (*white bars*), 125 (*grey bars*) and 250 ng (*black bars*) of plasmid per well per experiment in duplicates. Transfections with pMCV1.4-G_{VHSV} SV40 were included into each experiment to normalise results between experiments. *Expression in each dilution of plasmid constructs were normalised to the corresponding dilution of pMCV1.4-G_{VHSV} SV40×100 $P<0.05$. Data are depicted as the mean± one standard deviation from two different experiments, each performed in triplicate

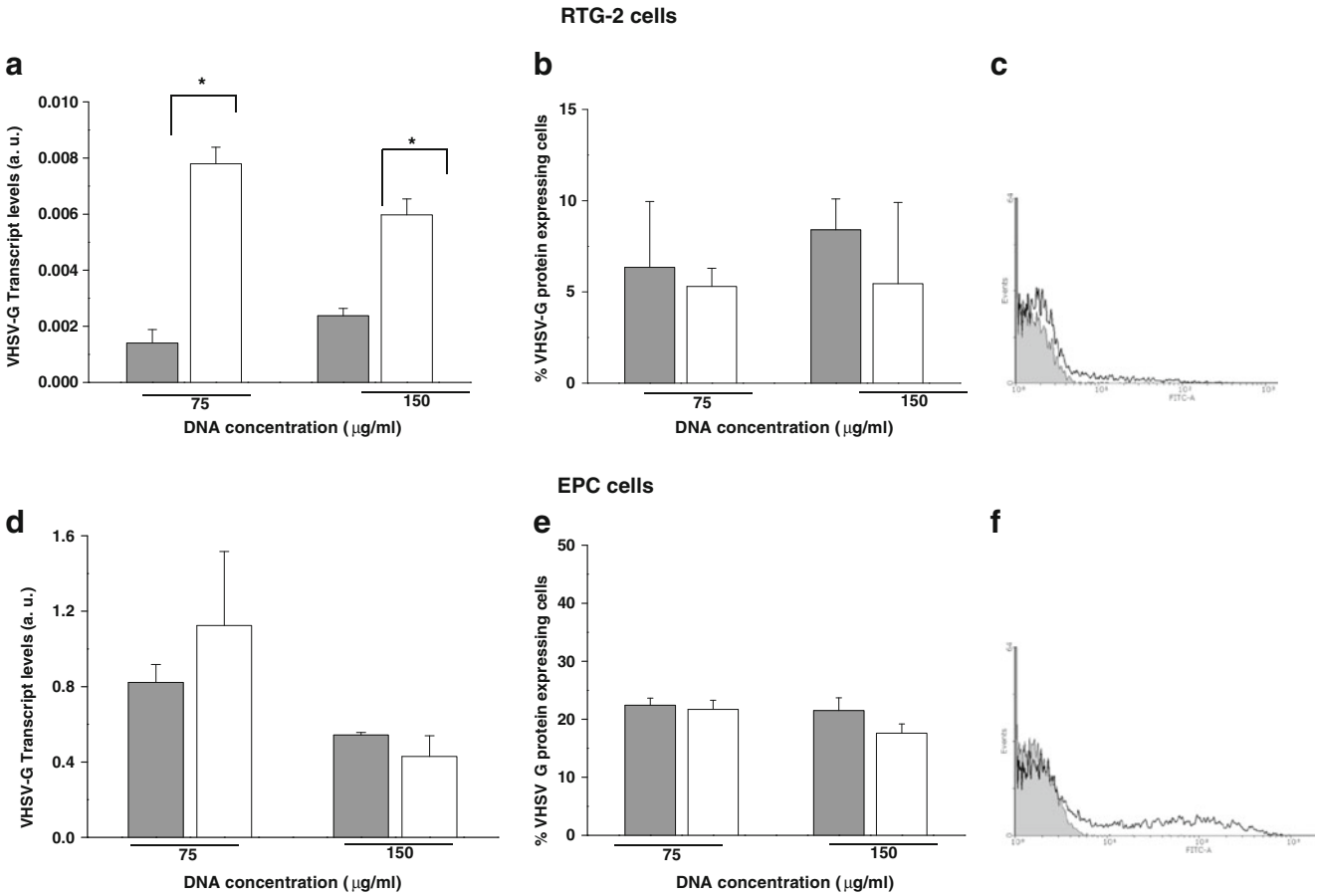


Fig. 3 VHSV G transcript and protein expression in RTG-2 (**a**, **b**, **c**) and EPC (**d**, **e**, **f**) fish cell lines. RTG-2 or EPC cells were transfected with pJAC-G or pAE6-G constructs. After 48 h (EPC) or 72 h (RTG-2), VHSV G transcript levels were estimated by qPCR (**a**, **d**) and G protein expression by flow cytometry (**b**, **e**) as described in 'Materials and methods' section. (**c**) and (**f**) show a representative merged histogram of the non-treated cells (*grey*) and the transfected cells (*black line*). Both plasmids at 150 ng/well or 75 ng/well. pJAC-G (*grey bars*) or pAE6-G (*white bars*). *a.u.* arbitrary units (VHSV G transcript expression relative to the housekeeping gene transcript expression). *Significantly different as determined by unpaired *t* test ($P<0.05$)

and Kongsuwan 1999). We corroborated that the G glycoprotein expressed from the pJAC-G vector in transfected cells was correct, by transfecting RTG-2 and EPC cells with pJAC-G and then stained them using a cocktail of anti-VHSV G MAbs (including conformational anti-gpG MAbs). The presence of the G glycoprotein was detected on the membrane of transfected RTG-2 and EPC (Fig. 4) cells.

Membrane fusion mediated by the G glycoprotein in pJAC-G transfected cells

Having established the correct expression and cellular localisation of the G glycoprotein, we also verified that the protein expressed from the pJAC-G vector in transfected cells was functional. Because one of the functional features of the glycoprotein G of rhabdoviruses is to mediate low-pH (5–6) dependent fusion, cell-to-cell fusion of G glycoprotein-expressing cells results in the formation of syncytia (multinucleated cells). Therefore, to examine the fusion properties of pJAC-G transfected cells, syncytium-forming assays were carried out. Syncytia were abundant in pJAC-G and pAE6-G transfected cells (Fig. 5), but differences in the syncytia average size were observed between transfected RTG-2 (seven to 10 nuclei per syncytia) and EPC cells (12 to 20 nuclei per syncytia) (not shown). Moreover, the percentage of nuclei in syncytia was higher in EPC (65 %) (Fig. 5b, d) than in RTG-2 cells (7 %) (Fig. 5a, c), correlating with the percentage of G expressing cells in each cell line (Fig. 4).

On the other hand, the percentage of nuclei in syncytia was higher in the RTG-2 cells transfected with pJAC-G than in those transfected with pAE6-G (Fig. 5a) suggesting that pJAC-G RTG-2 transfected cells express higher levels of VHSV-G protein per cell than the pAE6-G transfected ones. A representative image of the syncytia found in both lines is shown in Fig. 5c (RTG-2) and d (EPC).

In vivo G glycoprotein expression in muscle intramuscularly injected with pJAC-G

Rainbow trout were injected intramuscularly with pJAC-G and pAE6-G, and expression of the G glycoprotein at transcript and protein levels were evaluated 3 and 12 days post-injection (dpi) (Fig. 6). G glycoprotein transcripts were detectable in the muscle of all fish injected with the G-encoding plasmids. The accumulation of G glycoprotein transcripts was higher in the fish injected with pJAC-G at 3 dpi, but when injected with pAE6-G higher levels were observed at 12 dpi (Fig. 6a). The levels of transcription did not correlate with G protein levels, similarly to what occurred in vitro. G protein levels (Fig. 6b) were slightly higher in the muscle from fish injected with pAE6-G at days 3 and 12 post-immunisation than in fish injected with pJAC-G, but the differences between the two constructs were not significant.

Discussion

To date, the main emphasis on DNA vaccine research has been on their functionality and immunological responses whereas work on safety aspects has been deferred. In contrast, this work has mainly focused on one of the safety issues related to these vaccines, the presence of viral regulatory sequences in DNA vaccine vectors. To evaluate whether or not vector constructs harbouring regulatory sequences derived from genes homologous to the DNA vaccine target animal species could be a potential solution, we designed a new DNA vaccine vector containing

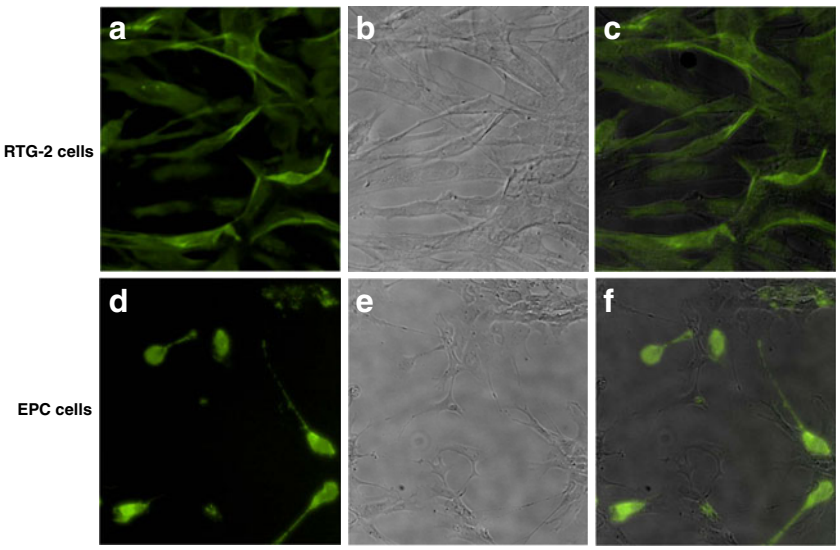


Fig. 4 Membrane immunofluorescence of the expressed G protein in RTG-2 (**a**–**c**) and EPC (**d**–**f**) transfected cells. **a**, **d** Fluorescent cell micrographs; **b**, **e** phase contrast; **c**, **f** merged fields

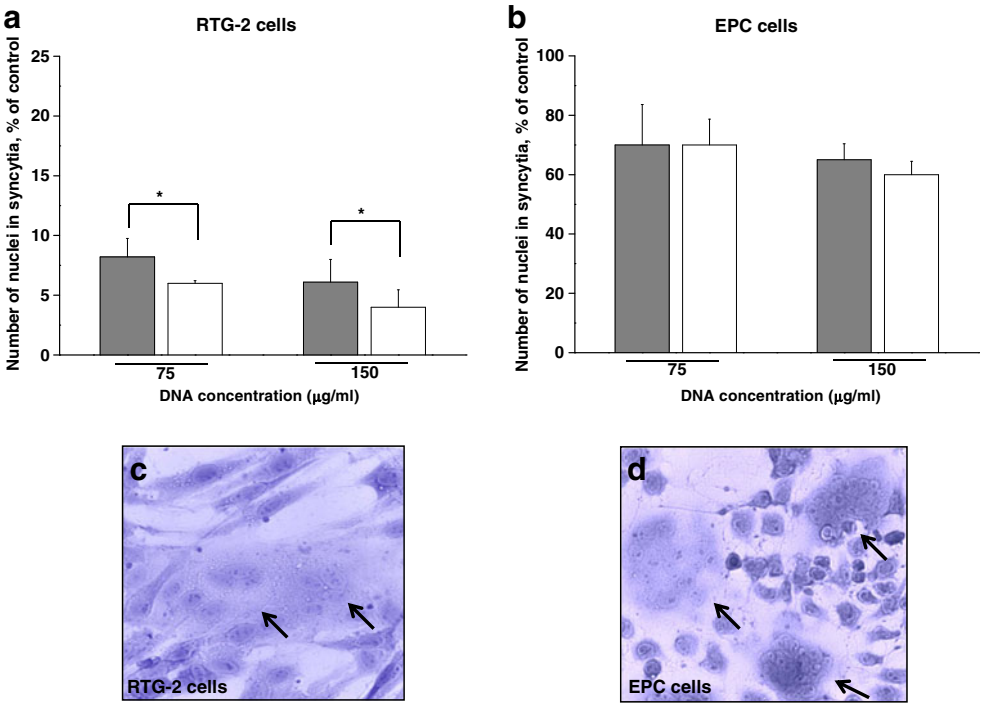


Fig. 5 Low-pH-induced fusion of pJAC-G and pAE6-G transfected RTG2 (**a**) and EPC (**b**) cells. To comparatively evaluate the functionality of the G expressed in RTG2 and EPC cells transfected with pJAC-G and pAE6-G, fusion was induced by low pH. Merged nuclei were counted and compared to the number of nuclei present in a 96-well plate. Both plasmids at 150 ng/well or 75 ng/well. pJAC-G (*grey bars*) or pAE6-G (*white bars*). *Arrows*—syncytia

regulatory sequences only from fish origin ('all-fish vector'). Then, with this new vector we develop an optimised DNA vaccine against the fish rhabdovirus, VHSV.

To develop such an all-fish vector, the previously described pAE6 vector containing the 5′ upstream sequences of the carp β-actin gene was chosen because in vivo fish DNA vaccination assays have shown that this vector conferred similar fish protection against VHSV lethal challenge than CMV-based vectors and therefore could be an effective alternative (Chico et al. 2009). Furthermore, data on the influence of transcription terminator sequences of fish origin in the expression of the G protein of VHSV have not yet been reported. Therefore, alternative transcription terminator sequences from fish genes had to be synthesised and then tested. The results showed that all-fish terminators exerted their function suitably and can be used to substitute the SV40 terminator. Among the seven transcription terminators tested, the one from zebra fish β-actin gene was selected to be combined with the pAE6 enhancer-promoter (pJAC-G), because (1) it worked in two different fish cell lines from cold as well as warm water fish and (2) it allowed the development of a homologue expression system by using both enhancer-promoter and terminator from a similar fish family.

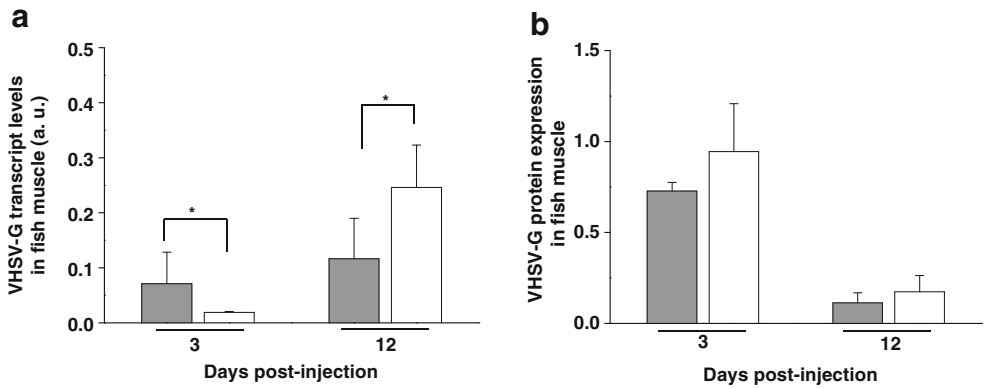


Fig. 6 VHSV G protein expression in skeletal muscle of rainbow trout injected with pJAC-G and pAE6-G. VHSV-G expression was studied in rainbow trout i.m. injected with 2 μg of pJAC-G or pAE6-G plasmids. After 3 and 12 days, transcripts and protein expression were analysed. Three and 12 days p.i., muscle samples from immunised fish were homogenised and analysed by ELISA using a non-conformation-dependent MAb to G_{VHSV}. Absorbance readings were measured at 450 nm. **a** Expression of G_{VHSV} transcripts in muscle tissue estimated by qRT-PCR. *Bars* represent the average values and standard deviations for three fish per group. **b** G_{VHSV} protein expression in muscle tissue estimated by ELISA. *Bars* represent the average values and standard deviations for three fish per group. pJAC-G (*grey bars*) or pAE6-G (*white bars*). *Significantly different as determined by unpaired *t* test ($P<0.05$)

The ability of pJAC-G to drive the in vitro expression of the VHSV G glycoprotein was evaluated in two different cell lines. On the one hand, since rhabdoviral infections begin on fish external surfaces (Costes et al. 2009; Encinas et al. 2010; Harmache et al. 2006; Helmick et al. 1995) and the epithelial fish tissue might be one of the main early targets for the replication of VHSV, the epithelial warm water EPC cell line was chosen. On the other hand, the possible use of DNA vaccines for salmonid fish would also require those vectors to be assayed in cold water salmonid cell lines such as the RTG-2 cell line derived from rainbow trout (*O. mykiss*) and therefore this cell line was also chosen. The G glycoprotein transcript levels were higher when both cell lines were transfected with the pAE6-G vector containing the SV40 terminator, suggesting an increased production and/or enhanced mRNA stability associated with the presence of viral terminator elements (Pfeiffer et al. 2010). Strikingly, the increased presence of G transcripts observed with pAE6-G was not always reflected on the amount of expressed G protein. This was observed in RTG-2 cells, where the percentage of G expressing cells was similar between the two constructs.

Since the expression of G might vary when plasmids are injected in vivo (Xiang et al. 1995), we also evaluated the expression of the G glycoprotein gene in the muscle of trout intramuscularly injected with pJAC-G and pAE6-G both at the transcript and protein expression levels. The results unequivocally demonstrated that the all-fish pJAC-G vector is a real alternative to conventional DNA plasmids.

In conclusion, this work shows that viral regulatory sequences might be replaced from a DNA vector without compromising the expression of the encoded antigen. However, this study is only a starting point due to the potential risk of insertional mutagenesis when highly interspecies-conserved sequences are used (Gregoriadis 1998; Smith and Klinman 2001). Consequently, to reduce this potential risk, we are also studying combinations of core and enhancer sequences of different fish promoters' terminator sequences to develop new synthetic regulatory sequences with lower homology to fish genomic sequences. It is expected that such all-fish vectors would contribute to make potentially safer fish DNA vaccines.

Acknowledgements Thanks are due to Beatriz Bonmati for technical assistance. This work was supported by Spanish projects AGL2011-28921-C03-01 and -02 and CONSOLIDER INGENIO 2010-CSD2007-00002 from MICINN and ISIC-2012-003 from Generalitat Valenciana.

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